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Tumorigenicity of *N*-Nitroso-Diaethyl, -Dimethyl and -Diphenyl-Amines in Skin Painting Experiments

A study utilizing the tetrazolium test and skin applications on hairless mice

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Abstract—*Groups of* 40 *hairless mice were painted for* 20 weeks *with single weekly applications of* 0.1 ml *of a* 1% *solution of* N-*nitroso-diaethyl-*, N-*nitroso-dimethyl-and* N-*nitroso-diphenylamine. The tumor yield was observed. After* 80 weeks *observation time, there were no papillomas, carcinomas or sarcomas developing on the skin or in other organs. However, in all three groups lung adenomas developed. Even among those* 40 *mice painted with* N-*nitroso-diphenylamine, three lung adenomas developed. The tetrazolium test for skin carcinogenesis was performed with the above-mentioned substances, and with* N-*nitroso-aethylurea. The latter substance gave a positive result, and it is known to be a skin carcinogen. The three other substances, which in this experiment were non-carcinogenic to the skin, gave negative results with the tetrazolium test. Thus, the study confirms the validity of the tetrazolium test to indicate skin carcinogens.*

INTRODUCTION

THE *N*-NITROSO compounds comprise several interesting chemical carcinogens with a high degree of organotrophy. Methyl-nitrosourea (MNU) and aethyl-nitrosourea (ENU) have been shown to be carcinogenic for the skin, but most of the other carcinogenic nitroso compounds have not been proven as skin carcinogens after topical application.

The tetrazolium test (TZT) was introduced by Iversen in 1962. It was claimed that it could indicate substances with skin carcinogenic potency. The TZT was previously shown to be positive for MNU [1] which is also a powerful carcinogen for mouse skin. It was therefore found of interest to test some other nitrosamines both with the TZT and with topical skin applications.

MATERIALS AND METHODS

Animals used

Hairless mice of the hr/hr Oslo strain were used. Spontaneous skin tumours or lung adenomas have not been observed in these animals, but reticuloses in internal organs sometimes occur in old animals [2].

All the mice were housed in plastic cages (8 animals in each) in a modern animal department with constant light/dark rhythms and were given a standard diet and water *ad libitum*. The cages were cleaned, and fresh water supplied at noon, three times a week.

Painting experiments

All the substances were applied in a 1% solution in acetone prepared in the cold. The solutions were divided into small batches which were kept in a deep freeze. Each week a new batch was opened and used.

For the painting experiments we used *N*-nitroso-dimethylamine (NDMA) from Eastman Organic Chemicals, Rochester, New York, U.S.A. *N*-Nitroso-diaethylamine (NDEA) and *N*-nitroso-diphenylamine (NDPA) were from Fluka, Buchs., Switzerland. For the TZT also *N*-nitroso-aethylurea (ENU) from Serva, Heidelberg, F.R.G., was used.

In the painting experiments, groups of 40 mice (16 males and 24 females) were given single weekly applications of 0.1 ml of a 1% solution of the substance to be tested (i.e.,

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1 mg per animal per week) on the interscapular area for 20 weeks. The animals were examined every week for a period of 80 weeks. Whenever possible, a careful necropsy was performed when an animal died, or at the end of the experiments, when all animals were killed. The lungs were fixed in formalin for 24 hr and then carefully palpated. All lesions felt (down to a size of 0.2×0.1 mm) were studied histologically.

TZT test

The tetrazolium test (TZT) was performed as described by Iversen [3]. Eight animals were used for each test. A skinfold from the back of the mouse was held in a special pair of forceps, and 0.02 ml of a 1% solution of MNU in acetone was dripped on the skin area inside the frame of the forceps. The acetone was allowed to evaporate in the stream of warm air from a hair-dryer. Twenty-four hr later the animals were killed and the skin removed. Each skin was fixed on a frame and incubated in a 1% solution of triphenyltetrazolium chloride in a buffer solution for 1 hr. The skin was then placed in a solution of ammonium chloride (1.48 mole/l, pH 9.5) and kept in the cold overnight. The next day the epidermis from the treated area was removed and a similar piece of epidermis from the opposite, untreated side of the back was used as a control. Two ml acetone were used to extract the red formazan produced by the epidermal cells. The degree of redness was measured photometrically. The epidermis was dried and its dry weight determined. The amount of formazan per mg epidermis in the treated area was compared with the same value from the control area.

RESULTS

Painting experiments

All painted animals showed signs of toxic effects on their skin, i.e., small ulcerations and scarrings. The mortality was small and the survival data are given in Table 1. However, no papillomas, carcinomas or sarcomas developed on the skin or in other organs (Table 1).

Of the 102 surviving animals, 20 had lung adenomas (Table 1), 18 had a single lung adenoma, one had two lung adenomas, and one had three adenomas. These two animals with more than one adenoma had both been painted with NDEE. Twenty adenoma-bearing animals, among 102 surviving painted animals, against none in a similar group treated with solvent alone [4] (see Discussion) is a significant result linking the occurrence of lung adenomas to the paintings ($\chi^2 = 22.174$, D.F. = 1, $P < 0.001$). The similar statistics for each of the three nitrosamines were:

NDEA: $\chi^2 = 14.118$, D.F. = 1, $P < 0.001$
NDMA: $\chi^2 = 5.2334$, D.F. = 1, $0.01 < P < 0.005$
NDFA: $\chi^2 = 3.1168$, D.F. = 1, $0.05 < P < 0.08$.

TZT tests

The results of the TZT are shown in Table 2. A result of the TZT higher than 1.2 is said to indicate a skin carcinogen. Carcinogenic potency was not indicated neither for NDEA, NDMA or for NDPA. There were no epidermal necroses or skin ulcerations after NDMA, which gave TZT values lower than 1.0.

A 2% solution of MNU gave a positive TZT result, indicating a carcinogenic substance.

DISCUSSION

NDEA has been shown to be carcinogenic in all animal species tested. It is a yellow,

Table 1.

Substance applied	Sex	No. of animals at start	No. of animals surviving	No. of animals with Skin tumours	No. of animals with Lung adenomas
N-nitroso-diphenylamine	M	16	14	0	3
	F	24	21	0	0
N-nitroso-diaethylamine	M	16	16	0	4
	F	24	14	0	8
N-nitroso-dimethylamine	M	16	14	0	2
	F	23	23	0	3
Sum	M	48	44	0	9
	F	71	58	0	11
	Total	119	102	0	20

Table 2.

Substance applied	Concentration (%)	Result of TZT
N-nitroso-diphenylamine	1	1.10
	2	0.99
N-nitroso-diaethylamine	1	1.05
	4	1.08
N-nitroso-dimethylamine	1	0.89
	4	1.02
N-nitroso-aethylurea	1	1.17
	2	1.44

volatile liquid. A survey of its chemical and physical properties and its carcinogenic potency is given in Vol. 17 of the IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans [4]. It induces benign and malignant tumours after administration by various routes, including ingestion, parenteral injection, inhalation and rectal instillation. The major target organs are the liver, respiratory and upper digestive tracts and kidney. After topical skin application to mice and hamsters, no local skin tumours have been observed, but squamous cell carcinomas from the mucosa of the nasal cavity were seen [5–8].

NDMA has been shown to be carcinogenic in all animal species tested. It is a yellow oily liquid. A survey of its chemical and physical properties and its carcinogenic potency is given in Vol. 17 of the IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans [4]. It induces benign and malignant tumours following administration by various routes, including injection and inhalation, in various organs in various species. It produces tumours of the liver, kidney and respiratory tract. Experiments with topical skin paintings have not been reported in the literature up to 1977.

NDPA is crystalline, appearing in olive-green flakes. It is widely used in the rubber industry to retard vulcanization. There are few reports in the literature reporting the testing for carcinogenicity [9–11]. It is not mentioned in the IARC Monograph [4] on nitrosamines, probably because these testings were largely negative, and from the viewpoint of chemical structure it has not been considered a carcinogen.

Quite recently, however, Cardy *et al.* [12] showed that chronic feeding of experimental animals with NDPA gave rise to bladder carcinomas in a high percentage of rats, but gave only non-neoplastic bladder lesions in mice. No lung adenomas were reported. Thus, it seems that NDPA is a weak carcinogen for rat bladder and the present study has added evidence of a tumorigenic potency for hairless mouse lung after topical skin applications. We saw no bladder tumours.

Both NDEA and NDMA are previously shown to be carcinogenic for the respiratory tract. The occurrence of lung adenomas is certainly a sign of tumorigenicity and is also usually accepted as a sign of carcinogenicity. Hence, the painting experiments showed that the substances were resorbed through the skin, and acted as lung tumorigens.

It is, of course, debatable whether the group of animals painted with the solvent alone in 1972 [2] is acceptable as a control group for statistical calculations. However, the author has for more than 20 yr used the hr/hr mice for carcinogenesis studies and performed autopsies on hundreds of mice treated with topical applications of benzene, acetone and carcinogens dissolved in these solutions. Lung adenomas have only been observed after carcinogen treatment, particularly after β-propiolactone and MNU [13].

The dose of the carcinogen reaching the lung after skin applications is probably low. If the control value is absolutely zero, however, even three adenoma-bearing animals among 40 (8%) is important. In the human situation, e.g., for an industrial carcinogen, tumours in 8% of the workers exposed would be a serious epidemic.

We have previously published results on the strong skin carcinogenicity of MNU and positive results obtained with the TZT [1]. Pelfrene [14] demonstrated that ENU is a skin carcinogen.

Thus, if one sums up the present results with TZT for some nitroso compounds, the following correlation has been shown:

	TZT	Skin carcinogenic potency
MNU	+	+
ENU	+	+
NDEA	−	−
NDMA	−	−
NDPA	−	−

This is a strong support for the value of the TZT to find skin carcinogens.

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